EXHIBIT B

Characterization of Heat Shock Protein 110 and Glucose-Regulated Protein 170 as Cancer Vaccines and the Effect of Fever-Range Hyperthermia on Vaccine Activity[1]

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Several studies have confirmed that certain stress proteins can function as potent vaccines against a specific cancer when purified from the same tumor. Recent studies of two long-recognized but unstudied stress proteins, heat shock protein (hsp) 110 and glucose-regulated protein (grp) 170, have shown them to be efficient peptide chain-binding proteins. The present investigation examines the vaccine potential of hsp110 and grp170. First, it is shown that prior vaccination with hsp110 or grp170 purified from methylcholanthrene-induced fibrosarcoma caused complete regression of the tumor. In a second tumor model, hsp110 or grp170 purified from Colon 26 tumors led to a significant growth inhibition of this tumor. In addition, hsp110 or grp170 immunization significantly extended the life span of Colon 26 tumor-bearing mice when applied after tumor transplantation. A tumor-specific cytotoxic T lymphocyte response developed in the mice immunized with tumor-derived hsp110 or grp170. Furthermore, treatments of the mice with bone marrow-derived dendritic cells pulsed with these two proteins from tumor also elicited a strong antitumor response. Last, we showed that mild, fever-like hyperthermic conditions enhance the vaccine efficiency of hsp110 as well as heat shock cognate 70, but not grp170. These studies indicate that hsp110 and grp170 can be used in hsp-based cancer immunotherapy, that Ag-presenting dendritic cells can be used to mediate this therapeutic approach, and that fever-level hyperthermia can significantly enhance the vaccine efficiency of hsps. *The Journal of Immunology,* **2001, 165: 490–497.**

Tumor-derived heat shock protein (hsp)[3]-peptide complexes (particularly hsp70 and glucose-regulated protein (grp) 94/gp96) have been demonstrated to serve as effective vaccines, producing antitumor responses in several animal models (1–4). This approach takes advantage of the peptide-binding properties of stress proteins that are responsible for their functions as molecular chaperones in numerous processes such as protein folding, transport, assembly, and peptide trafficking in Ag presentation (5–8). Indeed, since hsps purified from cells bind a spectrum of cellular peptides (9), purification of some stress proteins copurifies a cell-specific peptide "fingerprint" of the cell of origin. In the case of cancer cells, this presumably includes a subset of antigenic, tumor-specific epitopes. By virtue of these antigenic peptides, the hsp (or grp) preparation can be used as a vaccine. Vaccination with hsp-/grp-peptide complexes derived from tumors circumvents the need to identify a large number of CTL epitopes of a cancer and the technical limitations associated with that approach.

The hsps of mammalian cells can be classified into several families of sequence-related proteins. The most obvious mammalian hsps, based on protein expression levels, are cytoplasmic/nuclear proteins with masses of ~25 kDa (hsp25), 70 kDa (hsp70), 90 kDa (hsp90), and 110 kDa (hsp110). However, in addition to hsps, a second set of stress proteins has been long observed that are localized in the endoplasmic reticulum (ER). The induction of these stress proteins is not readily responsive to hyperthermic stress, as is that of the hsps, but is regulated by stresses, which disrupt the function of the ER (e.g., glucose starvation and inhibitors of glycosylation, anoxia and reducing conditions, or certain agents that disrupt calcium homeostasis). These stress proteins have been historically referred to as grps to clearly distinguish them as a group. The principal grps on the basis of expression have approximate sizes of 78 kDa (grp78), 94 kDa (grp94), and 170 kDa (grp170). grp78 is homologous to cytoplasmic hsp70, whereas grp94 is homologous to hsp90 (10, 11). Although individual stress proteins have been studied for several years (in some cases intensively studied, e.g., hsp70), the largest of the above hsp and grp groups, hsp110 and grp170, have been almost entirely ignored. These stress proteins have only been cloned within the last few years, and their characterization remains at a very preliminary level (12–16). Curiously, they have both been found by sequence analysis to represent large and highly "diverged" relatives of the hsp70 family. It is recognized today that the hsp70 "family," the hsp110 family, and the grp170 family comprise three distinguishable stress protein groups in eukaryotic cells that share a common evolutionary ancestor (11, 17). The existence of hsp110 in parallel with hsp70 in the cytoplasm and of grp170 in parallel with grp78 in the ER of (apparently) all eukaryotic cells argues for important differential functions for these distantly related protein families. Indeed, present data indicate important functional differences between these large and small stress protein groups; e.g., hsp110 appears to be significantly more efficient than hsp70 in binding peptide chain

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[3] Abbreviations used in this paper: hsp, heat shock protein; grp, glucose-regulated protein; hsc, heat shock cognate; DC, dendritic cell; Meth A, methylcholanthrene-induced fibrosarcoma; ER, endoplasmic reticulum; WBH, whole-body hyperthermia.

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but does not bind to ATP agarose, as does hsp70 (15, 18); grp170 binds peptide from TAP, whereas grp78 does not (19, 20).

Because of the above points and the previously demonstrated effectiveness of a few other stress proteins as vaccines, we undertook an analysis of effectiveness of the vaccine potential of hsp110 and grp170. In the present report, we describe the procedure for purification of hsp110 and grp170 and begin to evaluate their use as cancer vaccines using two mouse tumor models. In addition, we examined the use of hsp110 and grp170 in the preparation of dendritic cell (DC) anticancer vaccines. Finally, several recent studies indicate that fever-like therapy can have significant effects on several immunological end points. We also examine the effect of a fever-like thermal exposure on the effectiveness of these stress proteins as well as hsc70 as vaccines.

Materials and Methods

Mice and Abs

BALB/c mice (viral Ag free) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the mouse facilities at Roswell Park Cancer Institute. Abs to hsp110 and grp170 were made in our laboratory (13, 20). Abs to hsc70 were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Colon 26 carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Methylcholanthrene-induced fibrosarcoma (Meth A) was kindly provided by Pramod K. Srivastava (University of Connecticut School of Medicine, Farmington, CT) and maintained in ascites in BALB/c mice by weekly i.p. passage of 2 million cells.

Purification of hsp110, grp170, and hsc70

Both tumor tissue and culture cells were used for hsp isolation. A cell pellet or tissue (40–60 ml) was homogenized in 5 vol of hypotonic buffer (30 mM sodium bicarbonate (pH7.2) and protease inhibitors) by Dounce homogenization. The lysate was centrifuged at 4,500 × *g* and then 100,000 × *g* to remove unbroken cells, nuclei, and other tissue debris. The supernatant was further centrifuged at 100,000 × *g* for 2 h. Supernatant was applied to a Con A-Sepharose column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 15 mM 2-ME). The bound proteins were eluted with binding buffer containing 15% α-D-methylmannoside (Sigma, St. Louis, MO). For purification of hsp110, Con A-Sepharose unbound material was first dialyzed against 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 15 mM 2-ME and then applied to a DEAE-Sepharose column and eluted by salt gradient from 100 to 500 mM NaCl. Fractions containing hsp110 were collected, dialyzed, and loaded onto a Mono Q (Pharmacia) 10/10 column equilibrated with 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 15 mM 2-ME. The bound proteins were eluted with a 200–500 mM NaCl gradient. Fractions were analyzed by SDS-PAGE followed by immunoblotting with an Ab for hsp110, as described previously (21). Pooled fractions containing hsp110 were concentrated by Centriplus (Amicon, Beverly, MA) and applied to a Superose 12 column (Pharmacia), and proteins were eluted by 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 15 mM 2-ME with a flow rate of 0.2 ml/min. For purification of grp170, Con A-Sepharose-bound material was first dialyzed against 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl and then applied to a Mono Q column and eluted by a 150 to 400 mM NaCl gradient. Pooled fractions were concentrated and applied on the Superose 12 column (Pharmacia). Fractions containing homogeneous grp170 were collected. Hsp70 was purified as described previously (22). Con A-Sepharose unbound proteins were loaded on an ADP-agarose column (Sigma) equilibrated with binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 15 mM 2-ME, 3 mM $MgCl_2$, and protease inhibitors). The column was then incubated with buffer containing 5 mM ADP at room temperature for 1–2 h. Proteins were subsequently eluted with the same buffer. The elute was resolved on a fast protein liquid chromatography system using a Mono Q column and eluted by a 20–500 mM NaCl gradient. For purification of hsps or grps from liver, the 100,000 × *g* supernatant was first applied to a blue Sepharose column (Pharmacia) to remove albumin. All protein was quantified with a Bradford assay (Bio-Rad, Richmond, CA). In these studies, it should be noted that although grp170 was purified using a Con A-Sepharose column, contamination with Con A can be largely ruled out, because the protective immunity was only observed in mice immunized with tumor-derived grp170 preparations and not in normal liver preparations that also utilized Con A columns.

Immunoblot analysis

Equivalent protein samples were subjected to 7.5–10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) (21). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated for 2 h with primary Abs diluted 1:1000 in TBST. After washing, membranes were incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted 1:2000 in TBST. Immunoreactivity was detected using the ECL detection system (Amersham, Arlington Heights, IL).

Tumor rejection assays

Mice (6- to 8-wk-old female) were immunized s.c. with hsp110, grp170, or PBS twice at weekly intervals. Seven days after the second immunization, mice were challenged by s.c. injections of 20,000 Colon 26 tumor cells or intradermal injections of 100,000 Meth A tumor cells (viability of tumor cells is >99%). s.c. injections were administered in the flank area, and intradermal injections were given in the skin on the ventral aspect of the trunk. The shortest diameter (*A*) and the longest diameter (*B*) were measured with a caliper every 2 days to monitor tumor growth. The volume (*V*) was calculated using the formula $V = (A^2B)/2$.

Immunotherapy of mice bearing Colon 26 tumor

All mice were first inoculated s.c. with 500,000 live Colon 26 cells. After tumors were palpable and visible, mice were treated every week with PBS, liver hsp110 (40 μg), and tumor hsp110 or grp170 (40 μg). A total of five injections were performed during the protocol. The survival of mice was monitored and recorded as the percentage of mice surviving after the tumor challenge. Mice that appeared moribund were killed and seen as "not surviving."

Generation and assay of CTLs

Mice were immunized as described before. Ten days after the second immunization, spleens were removed and spleen cells (1×10^7) were cocultured in a mixed lymphocyte-tumor culture with irradiated (12,000 rad) tumor cells (5×10^5) for 7 days and supplemented with 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 50 μM 2-ME. Splenocytes were purified by Ficoll-Paque (Pharmacia) density centrifugation and used as effector cells. Cell-mediated lysis was determined in vitro using a standard ^{51}Cr release assay. Briefly, effector cells were serially diluted in 96-well V-bottom plates (Costar, Cambridge, MA) in triplicate with varying E:T ratios of 50:1 25:1, 12.5:1, and 6.25:1. Target cells (5×10^6) were labeled with 100 μCi of sodium [^{51}Cr]chromate at 37°C for 1–2 h. ^{51}Cr-labeled tumor cells (5000) were added in a final volume of 200 μl/well. Wells containing only target cells with either culture medium or 0.5% Triton X-100 served as spontaneous or maximal release controls, respectively. After a 4-h incubation at 37°C and 5% CO_2, 150 μl of supernatant was analyzed for radioactivity in a gamma counter and percentage of specific lysis was calculated by the formula: percent specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). The spontaneous release was <10% of maximum release.

Vaccination with DCs pulsed with hsps from tumor

Bone marrow was flushed from the long bones of the limbs and depleted of RBC with ammonium chloride. Leukocytes were plated in bacteriological petri dishes at 2×10^6/dish in 10 ml of RPMI 10 supplemented with 20 ng/ml murine GM-CSF (R&D Systems, Minneapolis, MN), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 mM 2-ME. The medium was replaced on days 3 and 6, and on day 8 the cells were harvested for use. The quality of DC preparation was characterized by cell surface marker analysis and morphological analysis. DCs (1×10^7/ml) were pulsed with tumor-derived hsps (200 μg) for 3 h at 37°C. The cells were washed and resuspended in PBS (10^6 pulsed DCs in 100 μl PBS per mouse) for i.v. injection. The entire process was repeated 10 days later, for a total of two immunizations per treated mouse. Ten days after the second immunization, mice were challenged with Colon 26 tumor cells (2×10^5).

Whole-body hyperthermia (WBH) exposure

Mice were first inoculated s.c. with 500,000 Colon 26 tumor cells on the flank area. After the tumor reached a size of ~1 × 1 cm, WBH was

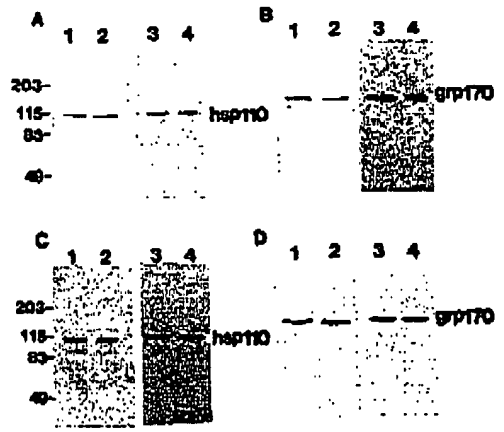

FIGURE 1. Hsp110 and grp170 preparations from tumor or liver of BALB/c mice. Hsp110 and grp170 purified from Meth A tumor (*top*), Colon 26 tumor (*bottom*, *lanes 1* and *3*), and liver of BALB/c mice (*lanes 2* and *4*) were separated by SDS-PAGE, followed by silver staining (*lanes 1* and *2*) or immunoblotting analysis (*lanes 3* and *4*) using Abs for hsp110 and grp170, respectively.

conducted as described before (22). Briefly, mice were placed in the microisolater cages preheated to 38°C that contained food, bedding, and water. The cages were then placed in a gravity convection oven (Memmert model BE500; Memmert, East Troy, WI) with preheated incoming fresh air. The body temperature was gradually increased 1°C every 30 min until a core temperature of 39.5°C (±0.5°C) was achieved. Mice were kept in the oven for 6 h. The core temperature of the mice was monitored with the Electric Laboratory Animal Monitoring System from Biomedic Data Systems (Maywood, NJ).

Results

Purification of hsp110 and grp170

Hsp110 and grp170 were purified simultaneously from tumor and liver. Purification protocols were developed as described in *Materials and Methods*, and homogeneous preparations for these proteins were obtained. The purity of the proteins was assessed by SDS-PAGE and silver staining as shown in Fig. 1. Approximately 20–50 μg hsp110 and 10–40 μg grp170 were obtained from each gram (wet weight) of tumor or tissue. The yield of grp170 from tumor is usually higher than that from normal tissue as a result of a higher level of grp170 expression in the tumor, possibly due to a hypoxic tumor fraction.

Hsp110/grp170 immunization causes the complete regression of Meth A tumors

We then investigated whether immunization with purified hsp110 and grp170 could protect mice against tumor challenge. For this purpose, the Meth A tumor model was initially used. We immunized mice twice with 40 μg (dose based on preliminary data) hsp110 or grp170 and then challenged them with Meth A cells by intradermal injection as described in *Materials and Methods*. Fig. 2 shows the results of this study. Separate lines present tumor growth data on individual animals, since some individual differences in the grp170-treated animals were observed. It is seen that mice immunized with hsp110 and grp170 were protected from the Meth A tumor challenge. Interestingly, and similarly to studies of others, most hsp110/grp170-vaccinated animals transiently developed tumors that then regressed and disappeared. However, in the mice that were immunized with grp170, two of five mice failed to develop any measurable tumor mass. To see whether this antitumor activity induces a long-term immunity against tumor, we challenged mice that survived with 100,000 Meth A tumor cells 5 months after the first challenge, and none of the mice was found to have developed tumor (data not shown).

Immunization of mice with tumor-derived hsp110 or grp170 leads to significant delays in growth of Colon 26 tumor

To test the generality of these observations on the vaccine activity of hsp110 and grp170 in the Meth A tumor system, we next chose the Colon 26 tumor model. This model was chosen since we found it to be generally resistant to various therapies. Groups of mice (five mice per group) were injected with PBS or with varying quantities of tumor-derived hsp110 or grp170 in 200 μl of PBS. These mice were then given booster injections 1 wk later. Hsp110 or grp170 was also isolated from the livers of the same animals, and this or PBS was used as control. Seven days after the last immunization, mice were injected s.c. on the right flank with 20,000 Colon 26 tumor cells. As seen in Fig. 3, all mice that were treated with PBS or liver-derived hsp110 or grp170 developed rapidly growing tumors. In contrast, mice immunized with hsp110 and grp170 from Colon 26 tumor showed a significant tumor growth delay, in general agreement with the above Meth A results. The inhibitory effect of hsp110 or grp170 vaccination on Colon 26 tumor growth was dependent on the dose of hsp110 or grp170 used for immunization. Although mice immunized with 20 μg (per injection) of hsp110 or grp170 showed an only slightly slowed tumor growth, those immunized with 40 or 60 μg of hsp110 or grp170 showed increasingly significant tumor growth delays (Fig. 3). Although tumor growth was not preventable in this highly aggressive

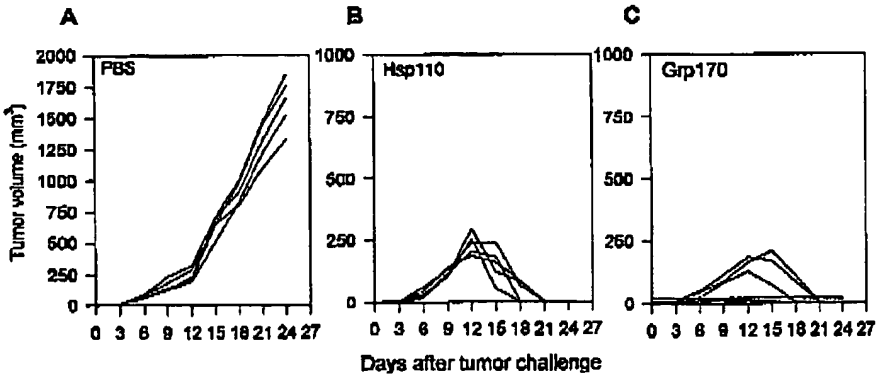

FIGURE 2. Immunization of mice with hsp110 or grp170 protects mice against Meth A tumor challenge. Mice were immunized s.c. with 40 μg of hsp110 or grp170 and boosted with the same amounts of these proteins 1 wk later. Seven days after the second immunization, the mice were challenged with 100,000 live Meth A tumor cells intradermally. Each group contained five mice, and each line represents the kinetics of tumor growth in one mouse.

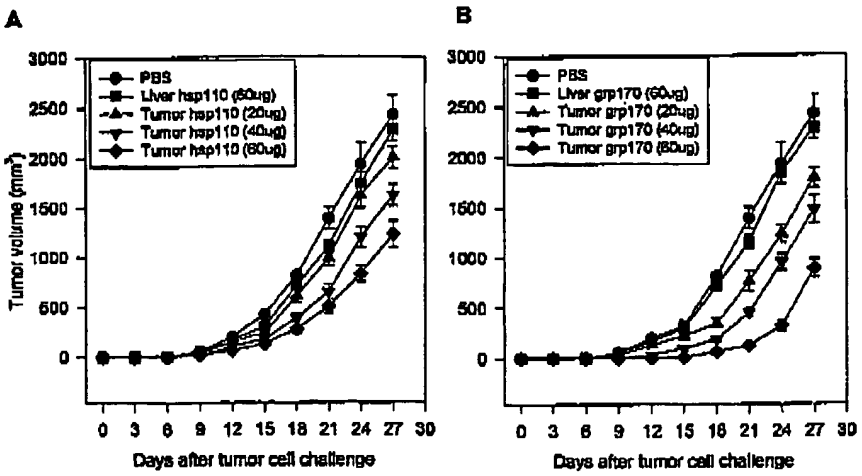

FIGURE 3. Immunogenicity of hsp110 and grp170 preparations purified from Colon 26 tumor. Mice were immunized twice with varying doses (20, 40, and 60 μg) of hsp110 and grp170 from Colon 26 tumor as indicated. Hsp110 or grp170 (60 μg) from liver of BALB/c mice was used as a control. One week after the second immunization, mice were challenged s.c. with 20,000 live Colon 26 cells.

and rapidly growing tumor system, these data demonstrate that hsp110 and grp170 have specific antitumor effects. On each day examined (e.g., 15, 21, and 27 days after challenge), the mean volumes of the tumors that developed in mice immunized with hsp110 or grp170 at doses of 40 and 60 μg were significantly smaller than those of control mice ($p < 0.01$, Student's t test). However, the differences in the mean volumes of the groups injected with PBS or liver-derived hsp110/grp170 preparations were not significant. Last, it was found that mice immunized with Meth A-derived hsp110 or grp170 were not resistant to challenge with Colon 26 tumor cells (data not shown).

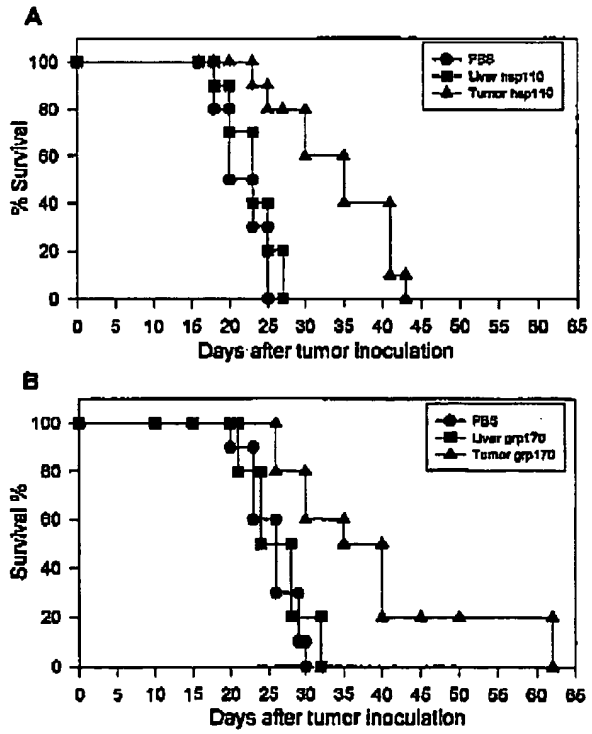

FIGURE 4. Effects of immunization with tumor-derived hsp on the survival of tumor-bearing mice. Mice were first inoculated s.c. with 500,000 Colon 26 cells. After the tumor was palpable, mice were treated with or without 40 μg of hsp110 or grp170 at weekly intervals. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge.

Hsp110/grp170 immunization improves the survival of Colon 26 tumor-bearing mice

In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, the aggressive Colon 26 tumor was again examined using a therapy approach. Tumor cells were transplanted into the flank of mice (10 mice in each group). When tumors were readily palpable after inoculation, animals were treated with liver- or Colon 26-derived hsp110 or grp170 on a weekly basis. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge. Tumor-bearing mice treated with autologous hsp110 or grp170 preparations showed significantly longer survival times compared with the untreated mice or mice immunized with liver-derived hsp110 or grp170. As shown in Fig. 4, all control mice died within 30 days, but approximately half of each group survived to 40 days and 20% of grp170-treated mice lived beyond 60 days, clearly demonstrating a beneficial antitumor effect. In parallel with the data shown in Fig. 2, these data suggest that grp170 is more efficient than hsp110 on an equal-mass basis.

Hsp110/grp170 vaccination elicits a tumor-specific CTL response

Since cellular immunity appeared to be critical in mediating the observed antitumor effects, we analyzed the ability of tumor-derived hsp110 and grp170 preparations to elicit a tumor-specific $CD8^+$ T cell response. Mice were immunized twice at weekly intervals with 40 μg of hsp110 or grp170 derived from Colon 26 or Meth A tumors. Splenocytes generated from these immunized mice were then cultured in vitro for 7 days with irradiated tumor cells. These cultured cells were then used as effector cells in the CTL assay. As shown in Fig. 5, a tumor-specific cytotoxicity was observed to occur against the tumor from which the immunogen (hsp110 or grp170) was derived. Splenocytes from mice immunized with Colon 26 cell-derived hsp110 or grp170 preparations showed specific lysis for Colon 26 tumor cells only, but not for Meth A tumor cells; conversely, splenocytes from animals immunized with Meth A tumor cells were only effective against Meth A

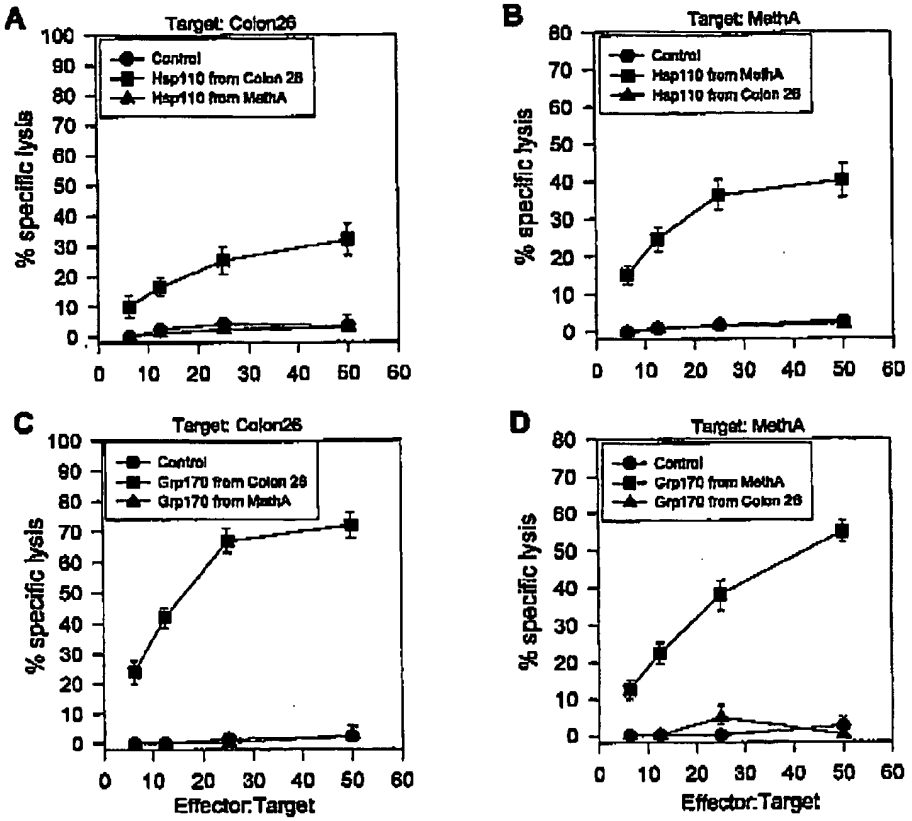

FIGURE 5. Tumor-specific CTL response elicited by immunization with tumor-derived hsp110 or grp170. Mice were immunized twice with PBS, hsp110, or grp170 (40 μg) at weekly intervals. One week after the second immunization, splenocytes were isolated as effector cells and restimulated with irradiated Colon 26 or Meth A tumor cells in vitro for 7 days. The lymphocytes were analyzed for cytotoxic activity using ^{51}Cr-labeled Colon 26 or Meth A cells as target cells.

cells and not against Colon 26 cells. This demonstrates that vaccination with hsp110 or grp170 elicits a tumor-specific CTL response. Splenocytes from naive mice were unable to lyse both target cells (control). Again, spleen cells derived from grp170-immunized animals yielded a greater percentage specific lysis than was obtained from hsp110-immunized animals.

Hsp110/grp170-pulsed DCs mount an effective antitumor response

To investigate whether APCs could be involved in the antitumor response elicited by hsp110 or grp170 immunization, we tested the ability of DCs to acquire an antitumor activity, presumably by presentation of hsp110- or grp170-chaperoned peptides. DCs were prepared from mouse bone marrow as described in *Materials and Methods*. DCs were then incubated with grp170 or hsp110 purified from the Colon 26 tumors for 3 h at 37°C. Cells were washed and resuspended in PBS. Pulsed DCs (10^6) in 100 μl of PBS were used for i.v. injection for each mouse. The entire process was repeated 10 days later. Ten days after the second immunization, mice were challenged with 2×10^4 Colon 26 tumor cells, and tumor growth was monitored by measuring the tumor diameter as shown in Fig. 6. It was observed that tumors grew rapidly in the mice that received PBS or (nonpulsed) DCs alone. However, tumor growth was significantly delayed in mice immunized with DCs pulsed

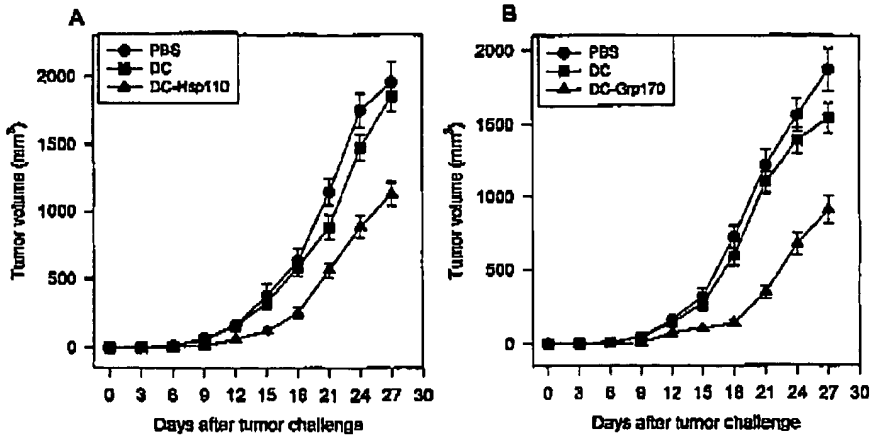

FIGURE 6. Immunotherapy with DCs pulsed with hsp110 or grp170. DCs (1×10^7) were generated from bone marrow of BALB/c mice and incubated with hsp110 or grp170 (200 μg/ml) in vitro for 3 h. DCs were washed and introduced to mice (10^6 cells in 100 μl PBS/mouse) by i.v. injection. The whole immunization process was repeated 10 days later. Mice were challenged with 20,000 Colon 26 cells 10 days after the second immunization.

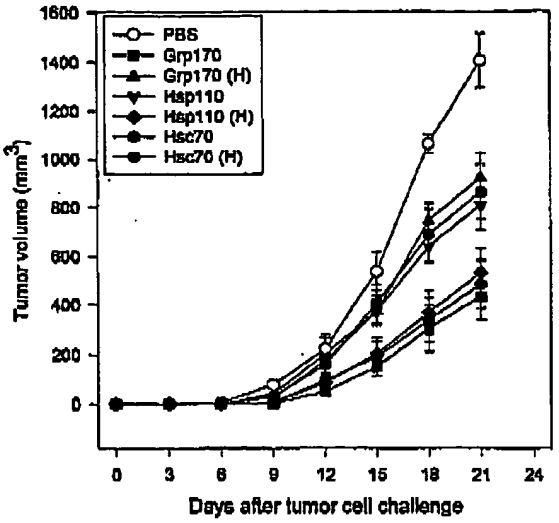

FIGURE 7. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70. Mice were first inoculated s.c. with Colon 26 tumor cells on the flank area. After the tumor reached a size of ~1 × 1 cm, WBH was conducted as described in *Materials and Methods*. Tumors were collected on the next day, and grp170, hsp110, and hsc70 were isolated. Mice were immunized twice at weekly intervals and then challenged with 20,000 live Colon 26 tumor cells.

with hsp110 or grp170. Grp170, once again, appeared to be more effective. Moreover, based on the immunization effects in the mice that received 10^6 DCs pulsed with 20 μg of protein and those that received two doses of 40 μg of protein by s.c. injections, it was found that less stress protein was required for DC-based immunotherapy.

Fever-like thermal conditions significantly enhance the efficiency of hsp110 or hsc70 as anticancer vaccines

Several recent studies have indicated that a modest increase in body temperature sustained for several hours, i.e., a condition comparable with common febrile response, can significantly affect certain immunological end points and immune function (22). We therefore exposed mice to 39.5°C (i.e., core temperature) WBH for a period of 8 h to determine whether hsp/grp vaccine efficiency might also be altered as a result of a fever-like thermal condition. Fig. 7 compares the effectiveness of hsp110 and grp170, as well as hsc70 (40 μg each), derived from Colon 26 tumors taken from both normothermic (control) animals and animals previously exposed to this fever-like thermal treatment. This figure illustrates several points. First, hsc70 or hsp110 is significantly more efficient when purified from tumors derived from animals receiving prior fever-range WBH. However, the prior fever-range thermal treatment is seen to reduce the vaccine efficiency of grp170. These data indicate that fever-like exposures can influence the Ag presentation pathway and/or peptide-binding properties of these two (heat-inducible) hsps purified from Colon 26 tumors but not a heat-insensitive grp. In addition to these observations, this figure also shows that grp170 purified from unheated control tumors (mice) is significantly more efficient in its vaccine efficiency when compared on an equal-mass basis with either hsc70 or hsp110 (without heat). This increased efficiency of grp170 compared with hsp110 is also reflected in the studies described above. This comparison is based on administration of equal masses of these proteins, and the enhanced efficiency of grp170 is further exacerbated when molecular size is taken into account (i.e., comparisons made on a molar basis). Third, hsc70 is seen here to be approximately equivalent in its vaccine efficiency (again, on an equal-mass but not equal-molar basis) to hsp110.

Discussion

It has long been recognized that the major hsps of mammalian cells are observed at 25–28, 70, 90, and 110 kDa, and other hsp families, e.g., hsp60 and hsp40, have been subsequently identified. These heat- or oxidative stress-inducible stress proteins principally reside in the cytoplasm and nucleus, excepting hsp60, which is in the mitochondria. It has also been long recognized that a second set of stress proteins called grps resides in the ER. This group of proteins is not responsive to typical heat shocks or oxidative stress but to reducing conditions (e.g., anoxia) or other states that interfere with the function of the ER. Principal grps have been observed at 78, 94, and 170 kDa. Both of the large stress protein species, hsp110 and grp170, have only recently been cloned. Their sequences have, surprisingly, shown them to be very large and greatly diverged relatives of the hsp70 family. They appear to possess many of the secondary structural features of hsp70 and are peptide chain-binding proteins. Although little is known about the cellular functions of hsp110, deletion mutational studies have defined its basic domains and indicate that it has a peptide-binding domain generally analogous to that of hsp70, while it also exhibits major functional differences from those of hsp70 (15, 18). Less is understood at the molecular level of grp170 structure and function; however, cellular studies have shown that it binds to Ig chain in the ER, may be the ATPase responsible for protein import into the ER, and actively binds peptides from TAP (i.e., the transporter associated with Ag processing; Refs. 11, 19, 20, 23, 24).

There is now considerable evidence from different laboratories that stress proteins (i.e., hsps and grps) can serve as vaccines that produce a tumor-specific CTL response and a protective antitumor immunity in animals (3–5, 25–29). We have examined here the capacity of hsp110 and grp170 to also function as stress proteins (or "heat shock") vaccines. We report that immunization with these two high molecular weight stress proteins leads to an antitumor immune response. It was found that hsp110 or grp170 immunization leads to a complete regression of Meth A tumor. In addition, either of these stress proteins was found to significantly inhibit Colon 26 tumor growth and significantly prolong the life span of mice with previously established tumors. These findings indicate that hsp110 and grp170 are both active anticancer vaccines.

Cytotoxicity assays described here demonstrate that hsp110 or grp170 immunization results in $CD8^+$ T lymphocyte response that correlates the in vivo tumor rejection observed. This is consistent with earlier studies concerning the antitumor immunity elicited by immunization with gp96 (25–29). In addition, the hsp-peptide complex, reconstituted in vitro, also elicits an Ag-specific CTL response (30). The capacity of hsp/grp to elicit an immune response is seemingly independent of the MHC type of the tumor, whereas the (presumed) presentation of the hsp-chaperoned peptides to CTL is MHC I restricted and is therefore defined by the MHC phenotype of the APC (9, 26, 31, 32). In addition, it is observed that priming of mice with Colon 26-derived hsp110 or grp170 only results in the lysis of Colon 26 tumor cells and not Meth A tumor cells. Conversely, a similar Meth A-targeted response was also obtained in the mice immunized with Meth A tumor-derived hsp110 or grp170. These observations are again consistent with earlier studies with other stress proteins showing that hsp immunization induces tumor-specific immune response (25, 28, 32, 33). Therefore, hsp-chaperoned peptides, even though

they are provided exogenously, are apparently capable of entering the class I Ag-presenting pathway. To investigate the molecular mechanism involved in hsp immunization-mediated antitumor immunity, additional experiments (i.e., T cell subset depletion) need to be performed.

DCs have been known to be highly specialized APCs and to be the principal activators of naive T cells in vitro and in vivo (34–37). Many have demonstrated that DCs pulsed in vitro with tumor Ag, tumor extracts, or mRNA (38–41) are capable of stimulating specific CTL activity and protect animals against subsequent tumor challenge. In the present study, we have shown that immunization with DCs pulsed with tumor-derived hsp110 or grp170 results in tumor growth inhibition in vivo, strongly suggesting that APCs are involved in the hsp-elicited antitumor response. It is suggested that hsp110- or grp170-peptide complexes can be targeted to APCs through a putative receptor. The hsp-chaperoned peptides are thus processed and re-presented by the MHC class molecules that stimulate Ag-specific $CD8^+$ T lymphocytes. Recently, it has been reported that hsp70 and gp96 receptors on the cell surface are involved in endocytosis of these stress proteins by APCs (42, 43). Further studies are needed to determine whether there exists a specific Ag internalization pathway mediated by these receptors and how hsp110- or grp170-associated peptides gain access to the ER of APCs.

Comparing the results of immunization of hsp110 and grp170 as immunogens in Colon 26 and Meth A tumor models and in the DC study, it is seen that grp170 appears to be more efficient than is hsp110 when administered on an equal-mass basis (i.e., Figs. 2–4). In addition, Fig. 7 further indicates that grp170 is also more effective on an equal-mass basis than is tumor-derived hsc70. We have also examined grp78, another relative of this stress protein superfamily. Curiously, grp78 appears to be largely ineffective as an anticancer vaccine when derived from tumors (data not shown). This latter observation is also consistent with data obtained by others (33). In this scheme, the approximate relative vaccine efficiency (least to most on an equal-mass basis for Colon 26 tumors) is as follows: grp78 (ineffective), hsp110 and hsc70 (similar effectiveness), and grp170 (most effective).

It has been shown that the immunogenicity of hsc70 can be attributed to the peptides chaperoned by it and that its properties as a vaccine are lost if the bound peptides are released (25, 45–47). Hsp110 and grp170 both appear to exhibit a peptide-binding cleft (11, 18, 44). However, hsp110 and grp170 differ dramatically from the hsc70s in their C-terminal domains, which, in the case of hsc70 proteins, appear to function as a "lid" for the peptide-binding cleft and may have an important influence on the properties of the bound peptide/protein and/or the affinity for the associated peptide/protein. Both hsp110 and grp170 appear to be more significantly efficient in binding to and stabilizing thermally denatured proteins relative to hsc70. This may reflect these structural differences and influence peptide-binding properties, a factor that is a key element in the ability of stress proteins to function as vaccines. Although hsc70 and hsp110 are approximately similar in vaccine efficiency, they may bind differing subsets of peptides (e.g., hsp110 may carry antigenic epitopes, which do not readily bind to hsc70); i.e., they may exhibit differing vaccine potential if not differing (mass) efficiencies. A similar argument can be made for grp170. The significant differences in molar efficiencies of these stress proteins may result from differing peptide-binding affinities, differing properties of peptides bound to each stress protein family, or differing affinities of APCs to interact with each of these four stress protein groups. It may also be noteworthy that grp170, the most efficient vaccine in this group, is the only gp.

Finally, reports in the last few years have suggested that a mild, fever-level thermal treatment can significantly stimulate various features of the immune response. At the cellular level, it has been shown that fever-like treatments of lymphocytes (39.5°C for 6–8 h) leads to activation of protein kinase C, massive cytoskeleton changes characteristic of a heightened activation status, and the induction of hsps including hsc70 and hsp110 (22, 48–49). In mice, fever-level hyperthermia has been shown to lead to an antitumor effect involving both the innate and specific immune systems (50). It is possible that mild hyperthermia, which is nontoxic, may lead to several changes in immunological parameters. We have shown here that the vaccine potential of hsc70 and hsp110 are significantly enhanced following fever-level therapy. This could result from enhanced proteosome activity, enhanced peptide binding of the hsp, altered spectrum of peptides bound to the hsp, or other factors. Since the hsps were purified 16 h after the 8-h hyperthermic exposure, the effect is maintained for some time at 37°C. It would seem that the factors leading to this enhanced immunogenicity would derive from an altered and/or enhanced antigenic profile of hsp-bound peptides. Stability following the hyperthermic episode suggests upstream changes in Ag processing that are still present many hours later, e.g., stimulation of proteosome activity. Another feature of fever-like hyperthermia is the highly significant induction of hsps in Colon 26 tumors (X.-Y. Wang and J. R. Subjeck, unpublished observations). Therefore, fever-like heating not only provides a more efficient vaccine in the case of the hsps examined, but also a lot more of it. Finally, it is intriguing that the observed increase in vaccine efficiency resulting from hyperthermia is seen only for hsp110 and hsc70. grp170, which is regulated by an alternative set of stress conditions such as anoxia and other reducing states, but not heat, is diminished in its vaccine potential by heat. It is not clear why grp170 efficiency as a vaccine is depressed by this heat shock condition. Further studies are required to determine how these changes arise.

Hsp vaccines are unique because of their promiscuous ability to chaperone and present a broad antigenic repertoire of tumor cell peptides. Thus, vaccination with hsps isolated from tumor cells circumvents the need to identify specific tumor Ags and hence extends the use of hsp-based immunotherapy to the majority of cancers of which specific tumor Ags have not yet been characterized (51). The administration of hsp/grp vaccines or hsp-/grp-pulsed DCs for cancer treatment might be safer than using whole tumor cell or cell lysates, specifically genetically modified cells, as tumor vaccines that could introduce transforming DNA or potentially immunosuppressive factors. The present study demonstrates that hsp110 and grp170 can both function as potent anticancer vaccines and provides strong additional supporting evidence for the development of hsp-/grp-peptide complexes as a basis for a new approach to cancer immunotherapy. Further investigation of mechanisms underlying the hsp-elicited antitumor response may help us to better understand the powerful immunological potential that is associated with hsp-mediated immunotherapy.

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